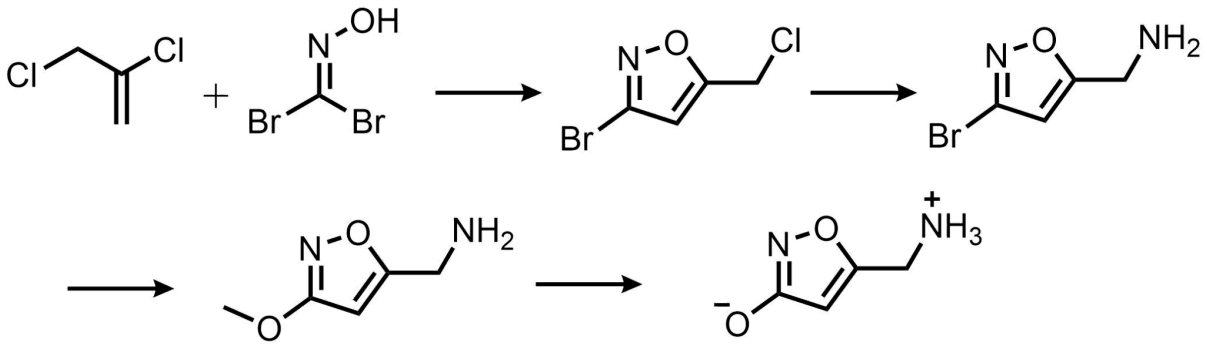
by arclightshroom

TOTAL SYNTHESIS OF MUSCIMOL, EXTRACTION AND QUANTIFICATION FROM AMANITA MUSCARIA

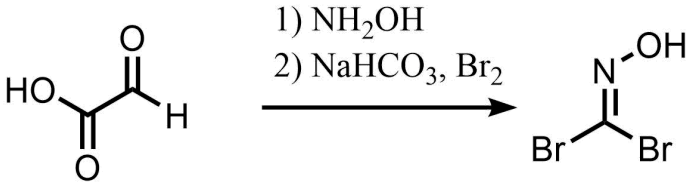
I've been fascinated by the Amanita muscaria mushroom since I was a kid, it always reminded me of a spirit of the woods, a white ghost with a big strange hat that could be found in the halloween period while wandering in the forest. As I grew more fascinated with chemistry and pharmacology the interest moved towards the chemistry and history of it's alkaloids, isoxazoles. Muscimol, the main psychoactive compound, is a GABA-A receptor agonist with sedating properties and a delirium-like "psychedelic" effect and it's the main chemical investigated. The original scope of the paper was to just make some muscimol, but after finding a big patch of Amanita mushrooms during a hike I took it as a sign and went more in depth. This works describes the original 1945 synthesis of the compound, a new approach to produce muscimol in high yields, the simplified extraction and isolation of the compound from fungal matter.

DBFO [3 + 2] cycloaddition route

This synthesis is the original improved method from 1945, which succeeded in making the isoxazole ring without organolithium compounds. This route also produces the intermediate 3-Meo-muscimol, which could be active on its own, but I've not yet explored. A sample has been saved for the future.



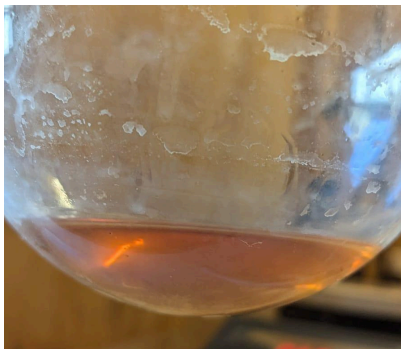
Preparation of dibromoformaldoxime (DBFO):



11.1g of a commercial 50% solution of glyoxylic acid in water are diluted with 28mL of distilled water, to the stirred solution was added a solution of 6.16g hydroxylamine sulfate in water (13mL). The solution was stirred at room temperature for one hour, after which it was neutralized to pH 4 with 12.2g of sodium bicarbonate. 60mL of DCM is added and everything is cooled in an ice bath. To the stirred biphasic mixture a solution of 24g bromine in DCM (30mL) is dripped in at a rate such that the temperature of the reaction mixture did not rise above 10° C. The pH is kept in the range of 2-4 with an additional 7.65g of sodium bicarbonate portionwise during the bromine addition. Upon completion of the addition of

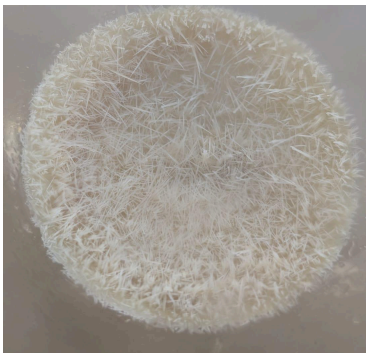
bromine, the solution was stirred for 3 hours at room temperature, the organic layer then separated, dried with sodium sulfate and desolventized to yield an orange oil which solidifies after a while in 9.5g crystals of DBFO. The crude product is recrystallized from 10mL of boiling hexane to yield 6g of pure DBFO, with a yield of 40%.

NOTE: The low yield I got vs the paper (71%) is probably attributed to my poor pH control, as during the bromine addition too much sodium carbonate made the pH temporarily way too basic with yield loss. If the product doesn't solidify on it's own, scrape it with a glass rod and let it sit in the freezer. The recrystallization step is important, a good quality material helps tremendously in the next reaction. Caution, DBFO is harsh on the eyes, they itched every time I opened the flask.



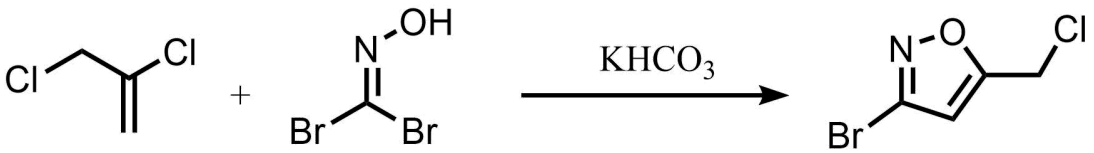
Liquid DBFO

Crude solid DBFO



DBFO after hexane recryst.

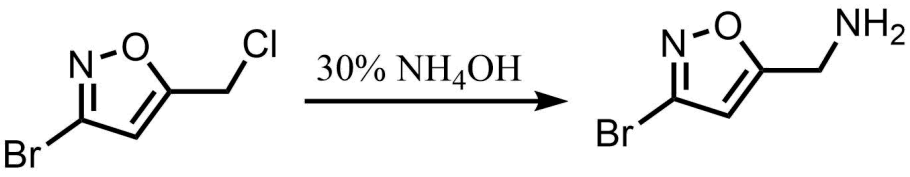
Building the isoxazole ring, 5-chloromethyl-3-bromo-isoxazole:



To a solution of 6.1g 2,3-dichloro-1-propene in 80mL ethyl acetate is added 6g of potassium carbonate and 8mL of water, the slurry is then stirred strongly for one hour. 6g of DBFO in 8mL ethyl acetate is added dropwise at room temperature, after the addition the mixture is stirred for 24 hours at room temperature. 30mL of water are added the organic layer then separated, dried with sodium sulfate and desolventized under vacuum to yield 5.33g of crude isoxazole product with a 50% yield.

NOTE: The crude material is fine for the next reaction, as the amine intermediates are far easier to clean. It can be distilled under vacuum, but I would consider doing it for larger quantities as I don't have appropriately sized glassware.

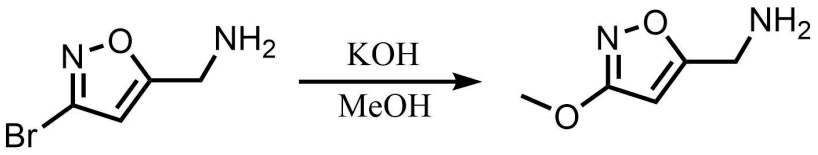
Preparation of 5-Aminomethyl-3-bromoisoxazole:



5.33g of 5-chloromethyl-3-bromo-isoxazole are dissolved in 65mL dioxane in an ice bath and 180mL of 30% ammonia are added dropwise at 0-5°C. After the addition the solution is stirred for 2 hours in ice then 4 hours at room temperature. The reaction mixture is distilled under vacuum to remove ~50% of the liquid then extracted with ethyl acetate. The organic layer was extracted with 10% HCl, which was then basified and extracted again with ethyl acetate. The solvent was dried with sodium sulfate and removed under vacuum to yield 0.8g of 5-Aminomethyl-3-bromoisoxazole, corresponding to a low yield of 16%.

NOTE: A methanol ammonia solution is probably more suited for this conversion. I'm also unsure of the solubility of this compound, and I fear it might be too water soluble to be just extracted with EtOAc. A better approach would probably be to reflux 5-chloromethyl-3-bromo-isoxazole in methanolic ammonia, rotovap away everything, dissolve in minimal EtOAc and use directly for the next reaction, or purify via column chromatography.

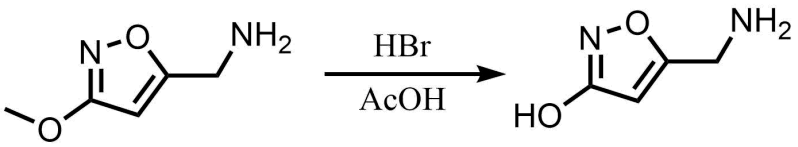
Preparation of 3-Meo-muscimol:



0.8g of 5-Aminomethyl-3-bromoisoxazole are dissolved in 18mL of MeOH:water (8:1) with 1.3g of KOH, the solution is then refluxed under nitrogen for 24h after which further 1.3g KOH is added and the reflux maintained for another 24h. Most solvent is removed under vacuum, 30mL of water is added and extracted with ethyl acetate. The organic layer was extracted with 10% HCl, which was then basified and extracted again with ethyl acetate. The solvent was dried with sodium sulfate and removed under vacuum to yield 0.84g of crude freebase. The product was dissolved in ethyl acetate and the hydrochloride salt precipitated with HCl gas, filtered and crystallized from boiling ethanol to yield 450mg of off white solid, corresponding to a freebase yield of 60%.

HCl crystals of 3-meo-muscimol

Preparation of muscimol:



350mg of 3-Meo-muscimol freebase are dissolved in 12mL of ~30% HBr in GAA and refluxed for 15 minutes. The mixture is then distilled dry under reduced pressure, the residue was then dissolved in a minimal amount of water and neutralized with ammonium hydroxide. After evaporating dry 96% ethanol was added to the residue and heated to boiling. Refluxing, water is added dropwise until the residue is dissolved, then more ethanol is added dropwise until the drop causes the solution to turn cloudy and then clear again. The solvent was then cooled at room temperature and then in the freezer, muscimol was obtained as a tan solid precipitate in 45% yield, 140mg.

NOTE: HBr in acetic acid is commercially available but expensive, it's way more convenient to make. HBr gas cannot be generated like HCl by dripping the aqueous acid into sulfuric acid, as oxidation to bromine occurs significantly at room temperature. Possible approaches are:
- Dripping bromine into tetralin produces pure dry HBr gas.
-Dripping acq. 47% HBr in hot, previously dehydrated phosphoric acid produces HBr gas (remember that concentrated hot phosphoric acid damages glassware)
-Making the solution by converting water in aqueous HBr into acetic acid with acetic anhydride. A maximum concentration of 15% can be achieved with this method, which is probably fine for this reaction.

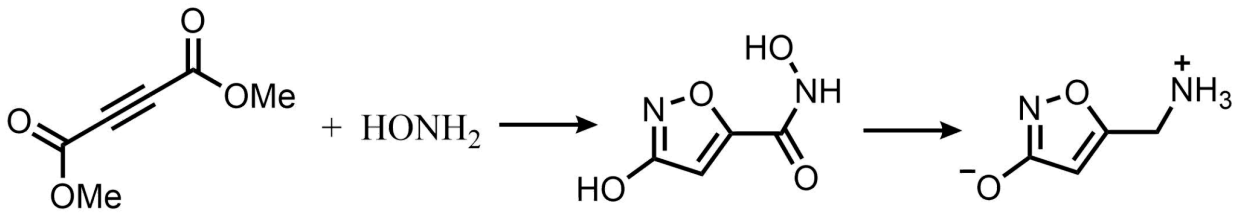
Crude muscimol after evaporation

Muscimol crystals clusters

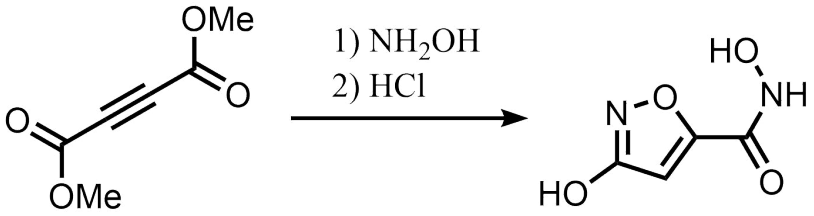
Apart from 2,3-dichloro-1-propene all the reagents used are cheap and easy to get, but the synthesis is far from optimal. The preparation of 5-Aminomethyl-3-bromoisoxazole is particularly dubious, I followed the paper, but cold aqueous ammonia seem too mild to accomplish that conversion in satisfactory yields, especially considering that other papers where the synthesis isoxazoles is discussed usually employ ammonia in methanol at refluxing conditions. This problematic reaction plus the fact that it's a 4 step synthesis resulted in a final yield of 2.16%, which looks more like the yield of an extraction, I wouldn't say that it's worth the effort of this process.

Hydroxamic acid route

This 2 step synthesis from a 2006 paper is the most convenient and promising procedure I have found for the production of muscimol in high yields.



Building the isoxazole ring, 3-Hydroxy-5-isoxazole hydroxamic acid:

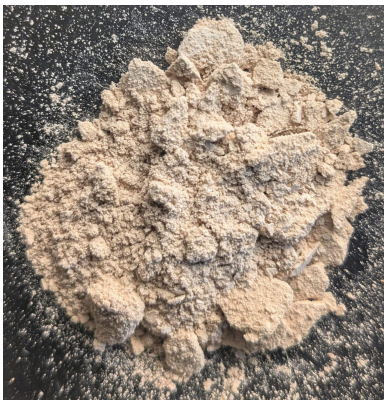


A flask containing a solution of 7g NaOH in 44mL of water was placed in an ice bath, hydroxylamine sulfate, 7.18g, was added portionwise keeping the temperature of the solution below 5°C. This solution was then maintained below 5°C while a solution of 5g dimethyl acetylenedicarboxylate in 2.5ml of methanol was added dropwise with rapid stirring. The solution is left stirring overnight at room temperature. After washing with 2x20mL DCM concentrated HCl is added to neutralize to pH 2-3, and the reaction mixture is evaporated dry under vacuum. The brown residue is then stirred with 80mL anhydrous ethanol for 30 mins, the solvent decanted from insoluble salts, and evaporated to afford crude 3-Hydroxy-5-isoxazole hydroxamic acid. Obtained solid was suspended with stirring in refluxing acetonitrile, cooled in the freezer, vacuum filtered and washed with more cold acetonitrile, to afford 2.8g of an off-white powder of acceptable purity. Final yield 56%.

NOTE: “Wet” ethanol can be used to dissolve the hydramic acid, but it will probably carry more salts impurity than the anhydrous solvent. Acetone can be used too, although in 3x quantity, as this compound is less soluble in it

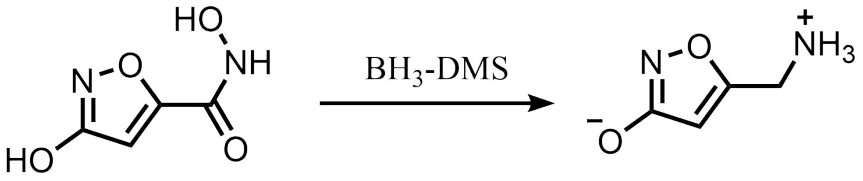
Post reaction mixture crude

After EtOH treatment



After ACN washing

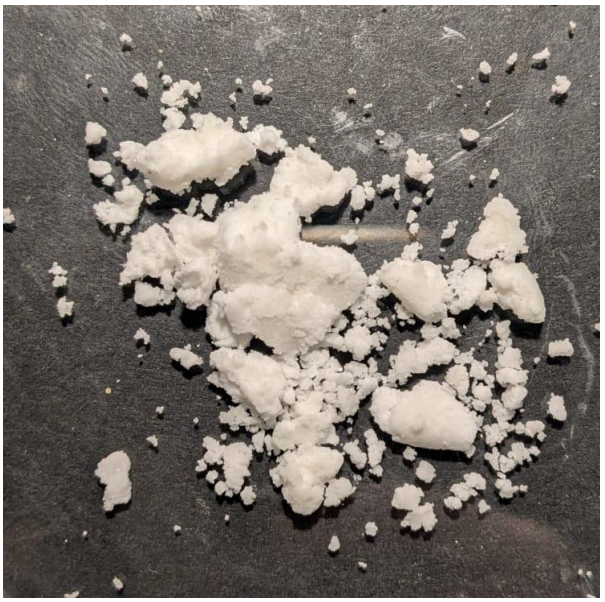
Preparation of muscimol:



1.85g of dry and finely powdered 3-Hydroxy-5-isoxazole hydroxamic acid are suspended in 60mL dry THF. The 2 neck reaction flask is equipped with a septum and a reflux condenser, attached to the Schlenk line, evacuated and kept under nitrogen flow. With strong stirring in an ice bath, 11mL of borane dimethylsulfide complex is injected portionwise through the septum over 40 minutes, keeping the temperature at 0-5°C. The resulting white suspension is refluxed for 22h, then cooled to 5°C with an ice bath and neutralized with careful addition of 15mL anhydrous methanol through the septum. After stirring at room temperature for 1 hour to make sure all the borane has been quenched, a gentle stream of HCl gas is bubbled through the solution for 2 hours to destroy the amine-borane complexes formed. The reaction mixture is refluxed for 1 more hour then the volatiles distilled off at normal pressure. The thick sludge remaining is dissolved in 20mL of deionized water, washed with DCM and added to 80g strongly acidic ion exchange resin on a column (stationary phase: cation [H+/Na+] exchanging ion resin, sulfonic acid functionalized) and washed with deionized water to neutrality. After only clear neutral water came off the column the product is eluted with 2M ammonium hydroxide. Evaporation gives 1.13g of muscimol in a 77% yield, further purification can be done with recrystallization from ethanol/water, but the purity was considered satisfactory.

NOTE: Unfortunately neither KH or LAH work for this reduction, and a reactive borane specie must be used. Out of the options borane DMS is the safest, as it's more stable in oxygen and water and less likely to spontaneously combust while maintaining high reactivity. The use of a Schlenk line is an overkill but I wanted to be extra safe as this was my first time handling borane complexes, a second test without a line proved successful. 3 cycles of vacuum/nitrogen with a 2 way adapter and a nitrogen balloon is sufficient, although this was tested on a really small scale and I'd be careful scaling up.
Once borane has reacted dimethylsulfide is left, and while not being particularly toxic it has an extremely powerful stench that can be smelled at 0.02ppm, so it must be dealt with properly to avoid the whole neighborhood smelling like rotten cabbages. The trap (attached to the gas exhaust of the Schlenk line in my case) is made of 3 gas washing bottles in serie, one empty to prevent suckback in case of a system failure, the middle one with concentrated bleach and the last one with activated carbon. Before HCl gas treatment the bleach one is swapped for a sodium bicarbonate solution to quench the acid and avoid chlorine generation, and swapped back after. When distilling away the volatiles, keep the traps connected to the vacuum port of the distillator, and collect everything that boils before THF (dimethylsulfide boils at 37°C) in a flask pre-filled with bleach. Swap the receiving flask with an empty one when THF comes over. After the reaction soak every piece of glassware in bleach, preferably remaining inside the fume hood/ventilation system in place.
Neutralization with methanol must be done EXTREMELY carefully, it's violent and releases a ton of hydrogen.

Crude muscimol HCl



Muscimol

Extraction and isolation of muscimol from fungal matter

Muscimol is an interesting and obscure drug with a fascinating history, unfortunately, it can't be found for sale pure and the synthesis is prohibitively complex for someone without extensive chemistry knowledge. This section describes a method for the extraction and isolation of muscimol from Amanita muscaria, made to be easy and direct to be followed by anyone interested in the compound but lacking chemistry knowledge.

The mushrooms I foraged for this experiment

Fresh Amanita muscaria mushrooms are dried in a desiccator and blended to afford 31.2g of dry powder, which was added to a flask with 150mL of deionized water and the pH adjusted with concentrated HCl to 2-3. The stirring suspension was refluxed for 3h, then vacuum filtered while still hot to remove fungal matter and brought back to boiling without condenser to allow concentration to about 50mL. Once cooled to room temperature the solution is

washed with 3x10mL ethyl acetate and then loaded in a column filled with 40g strongly acid ion exchange resin. The column is washed with deionized water until neutrality, then eluted with 2M ammonium hydroxide. The basic extracts are reduced dry under vacuum to a reddish goop, attempts to solidify it failed, a crystallization attempt was made by dissolving the goop into minimal distilled water and leaving it out to slowly evaporate in the course of a few days. 233mg of brown solid crystals were recovered.

NOTE: If a vacuum filtering setup is not available, break the dry mushroom into larger pieces by hand, as fine powder is optimal for extraction but a pain to gravity filter. The best way without vacuum is to extract larger chunks, filter through cloth and squeeze after to get everything out (wear gloves).
Boiling the mushroom in acidic water is the well known method for its preparation, so if the goal is making a tincture to consume directly use food safe acids like acetic or citric. HCl is the best option for isolation via ion exchange, but not the safest for direct consumption.
The ion exchange resin used is nothing fancy, just standard resin for water purification. Make sure it's strongly acidic functionalized with sulfonic groups.
Boiling the ammonium hydroxide solution to dryness at standard pressure degrades a lot of the product, use vacuum distillation if available, otherwise let it evaporate in a closed container with a drying agent. Remember to keep the compound away from sunlight, UV light oxidizes isoxazoles over time.
233mg of muscimol for 30g of dried mushroom is way too much according to literature muscimol % content, impurities are likely to be betalamic acid derivatives pigments (all zwitterionic and likely to follow muscimol in the column).

Muscimol crystals after slow evaporation

Analytical section

Each step of the synthesis was analyzed by TLC and melting point (when possible) to quickly determine the outcome of the reaction; eluents, Rf values and melting points are reported here.

Melting points:

COMPOUND	OBSERVED M.P	LITERATURE M.P
Dibromoformaldoxime	67°C	67°C
5-Aminomethyl-3-methoxyisoxazole HCl	166°C	175°C

(3-Meo-muscimol HCl)		
3-Hydroxy-5-isoxazole hydroxamic acid	200°C	203°C
Muscimol	184°C	185°C

TLC analysis:

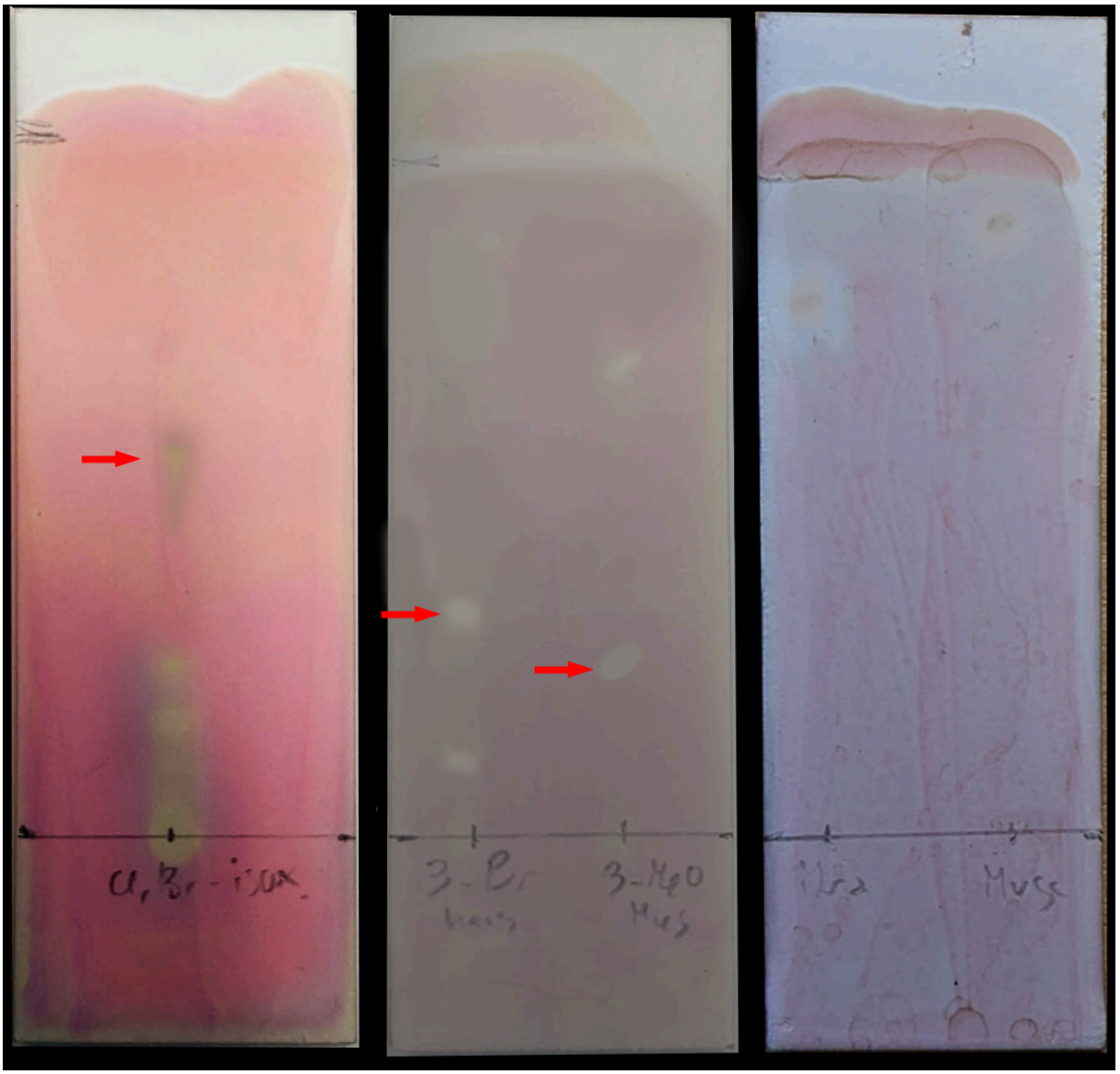


Plate 1, 5-chloromethyl-3-bromo-isoxazole:

- Eluent: cyclohexane:EtOAc (90:10), Rf: 0.58
- Tar is present at starting line, along with 2 impurities with Rfs 0.17 and 0.25

Plate 2, 5-aminomethyl-3-bromo-isoxazole:

- Eluent: DCM:MeOH:30% ammonium hydroxide (95:5:0.5), Rf: 0.32
- Impurity with Rf 0.12 and smear near solvent line at 0.74

Plate 2, 3-Meo-muscimol:

- Eluent: DCM:MeOH:30% ammonium hydroxide (95:5:0.5), Rf: 0.28
- Impurity with Rf 0.64. Maybe the low yield in this reaction derives from the dirty staring materials. Column chromatography or distillation in the previous steps might increase yield.

Plate 3, 3-Hydroxy-5-isoxazole hydroxamic acid:

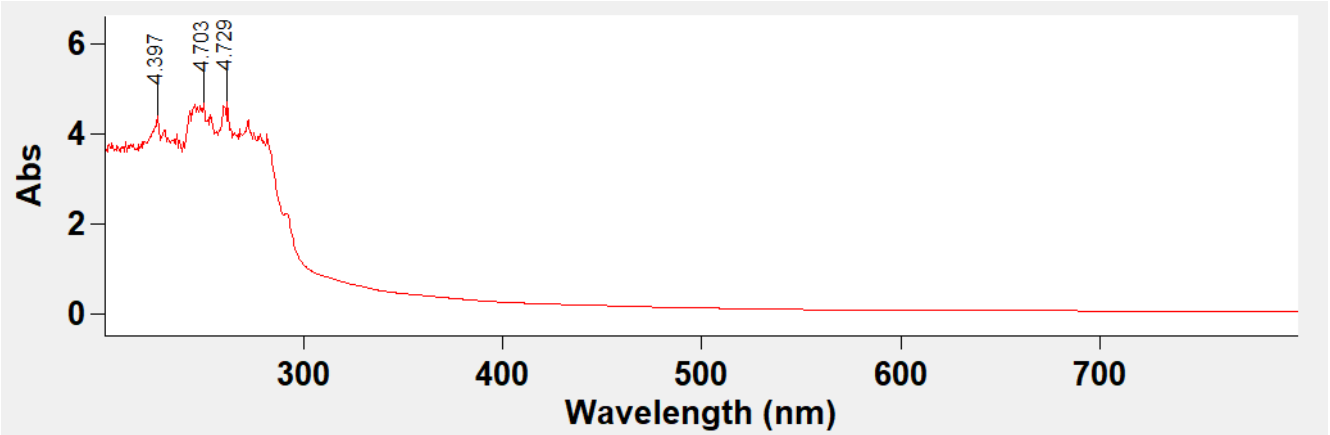
- Eluent: n-BuOH:AcOH:H2O (60:20:20), Rf: 0.78

Plate 3, muscimol:

- Eluent: n-BuOH:AcOH:H2O (60:20:20), Rf: 0.9

Plates visualized with potassium permanganate staining.

UV-Vis (Abs.) spectra of muscimol:



Measured at 1 mg/mL in water

Absorbance peaks at 260nm, 250nm and 210nm

Sources

1) An Improved Synthesis of Muscimol. (1992). In Synthetic Communications (Vol. 22, Issue 13, pp. 1939–1948). Informa UK Limited. https://doi.org/10.1080/00397919208021324

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